(D-ALA^2, N-VAL^5), (D-ALA^2, MET^5) ENKEPHALINS AND ENKEPHALINAMIDES

Correlation between biological activity and NMR features

J. E. GAIRIN, R. HARAN, H. MAZARGUIL+, Y. AUDIGIER+ and J. Cros+

Laboratoire de Chimie de Coordination and +*Laboratoire de Pharmacologie et de Toxicologie Fondamentales du CNRS, Associés à l'Université Paul Sabatier, 205, route de Narbonne, 31078 Toulouse Cédex, France*

Received 18 February 1981; revised version received 8 April 1981

1. Introduction

Recent research on the properties of enkephalins has expanded considerably due to interest in the properties of analogues of the two natural pentapeptides. Most synthetic analogues show analgesic activity, but their potency varies considerably depending on the nature and the position of the substitutions [1–3]. Following the demonstration of the heterogeneous nature of the opiate receptor populations, we have studied the relationship between the chemical structure of the enkephalin analogues and the selectivity for each type of receptor [4]. The results show that (D-Met^2, N-Val^5) enkephalinamide and (D-Ala^2, N-Val^5) enkephalinamide are potent agonists of opiate and enkephalin receptors, more active than the chemical isomers containing (Pro^5) [5]. Furthermore, (D-Ala^2) analogues can discriminate between opiate μ receptors in guinea-pig ileum (GPI) and the predominantly δ receptors in mouse vas deferens (MVD) [4]. We now report a correlation between strong δ receptors selectivity or binding and the structure induced in the tyrosine residue and in the peptide chain by substitution at position 5, as seen by NMR.

2. Materials and methods

2.1. *Chemical synthesis*

The enkephalin analogues were prepared as in [5]. The degree of purity was checked by thin-layer chromatography on silica-gel plates (ref. Merck 5626) in BuOH:AcOH:H_2O (4:1:1) as solvent. R_F are given in brackets: Tyr–D-Ala–Gly–Phe–N-Val-NH_2 (0.70); Tyr–D-Ala–Gly–Phe–N-Val-OH (0.74); Tyr–D-Ala–Gly–Phe–Met-NH_2 (0.68); Tyr–D-Ala–Gly–Phe–Met-OH (0.73).

2.2. *Preparation of samples for ^1H NMR studies*

The samples were prepared by dissolving 6 mg (~10 μmol) of enkephalin analogues in 0.7 ml $(CD_3)_2SO$ (99.9% D), reaching ~14 mM. ^1H spectra were recorded on a CAMECA 250 spectrometer operating at 250 MHz. All chemical shifts are given with respect to tetramethylsilane, $(CH_3)_4Si$, which was used as an internal standard in all samples.

2.3. *Biological activity on isolated organs*

Isolated GPI was placed in organ baths containing Krebs solution and then stimulated by field stimulation (0.1 Hz, 0.1 ms, maximal voltage). The longitudinal contractions were recorded isometrically.

The agonist potency (inhibitory effect) of enkephalin analogues were tested by cumulative dose–response curves as in [6]. Isolated MVD was prepared and used as above and in [7].

3. Results

Fig.1 shows the proton NMR spectra of (D-Ala^2, N-Val^5-OH) and (D-Ala^2, N-Val^5-NH_2) in DMSO-d_6 solutions containing trace amounts of water. Unambigous proton assignments were obtained from selective irradiations performed at different temperatures in order to avoid overlaps and from comparisons of amide proton chemical shifts in preliminary separated studies of di- to tetrapeptides sequencing the analogues (e.g., Tyr–D-Ala, Tyr–D-Ala–Gly and Tyr–D-Ala–Gly–Phe) [8]. Furthermore, the NH resonance of

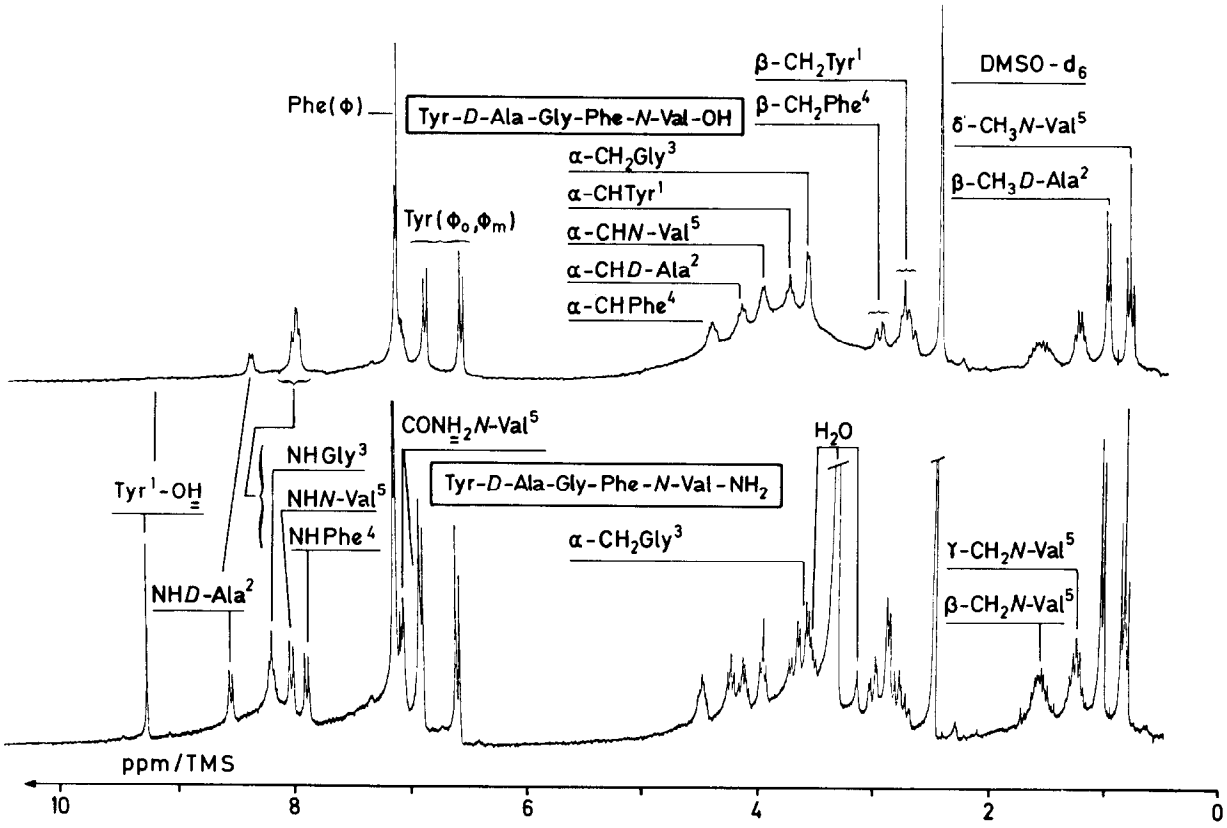

Fig.1. ^{1}H NMR spectra of (D-Ala^2, N-Val^5) enkephalin (above) and enkephalinamide in DMSO-d_6.

D-Ala^2 is easily assigned, based on the fact that the broadening of the signal with increasing temperature is the result of the proximity of the NH_3^+ moiety [9].

The water protons only appear as a broad slightly shifted absorption in the case of (D-Ala^2, N-Val^5-OH) and as a sharp line in the case of the amide analogue. Furthermore, the phenolic proton is only seen for the amide, at δ = +9.40 ppm. The free acid form has overlapping NH amide resonances of the Gly^3, Phe^4 and N-Val^5 residues near $\delta \sim 8.1$ ppm. The amide form, on the other hand, shows well separated peptide NH resonances. In both cases, the D-Ala^{2}NH is significantly shifted to lower fields. Other major differences can be noted in the amide compound spectrum, such as the resolution of the Gly^3 ABX pattern near $\delta \sim +3.6$ ppm, and the modifications of $^3J_{NH-H\alpha}$ (table 1). The C ter-

Table 1
$^3J_{NH-H\alpha}$ (Hz) coupling constants and for (D-Ala^2, N-Val^5) the temperature dependence coefficients θ (ppb/degree)

	D-Ala^2		Gly^3		Phe^4		N-Val^5		Met^5
	J	θ	J	θ	J	θ	J	θ	J
D-Ala^2, N-Val^5-OH	7.7	3.88	4.8[a]	2.03	–[a]	1.74	7.4[a]	4.25	–
D-Ala^2, N-Val^5-NH_2	7.3	2.70	4.4	2.51	8.5	3.17	8.1	4.22	–
D-Ala^2, Met^5-OH	8.1		4.8		8.1		–		6.6
D-Ala^2, Met^5-NH_2	6.6		5.3		8.1		–		8.1

[a] Approximated (Gly^3 and N-Val^5) or unresolved (Phe^4) values

Table 2
Activity of enkephalin analogues on guinea-pig ileum (GPI) and mouse vas deferens (MVD)

Compound	GPI IC_{50} (nM)	MVD IC_{50} (nM)	GPI / MVD
(D-Ala^2, Met^5)-Enkephalin	114 ± 23 (3)	1.3 ± 0.2 (3)	87.7
(D-Ala^2, Met^5)-Enkephalinamide	39 ± 4 (3)	6.3 ± 0.8 (5)	6.19
(D-Ala^2, N-Val^5)-Enkephalin	160 ± 11 (3)	2.4 ± 0.2 (3)	66.6
(D-Ala^2, N-Val^5)-Enkephalinamide	40 ± 5 (6)	6.9 ± 1.1 (6)	5.8

minal amide NH_2 resonances are not readily apparent in fig.1, but are located under the tyrosine and phenylalanine complex spectra with the help of variable temperature probe showing upfield shifts as temperature increases.

The same general features are observed on the ^1H NMR spectra of (D-Ala^2, Met^5-OH) and (D-Ala^2, Met^5-NH_2). Assignments obtained from selective irradiations agree well with [10] using (D-Ala^2, Met^5-OH).

The results in table 2 show that the enkephalin analogues are more active on MVD than GPI, bearing up their better activity on δ receptors. Whatever the nature of the residue on position 5 (Met or N-Val), the presence of the amide group on the C-terminal carboxylic function leads to an enhanced activity in the GPI assay, whereas opposite results are observed in the MVD assay where the amide derivatives are shown to be less active than the acid forms. The ratio IC_{50} (GPI)/IC_{50} (MVD) is therefore considerably modified by the amidation of the C-terminal residue which suggests a differential effect on the binding to the 2 types of opiate receptors present in these bioassays.

4. Discussion

The comparative analysis of the chemical structure and the biological activity have been useful to determine the critical segments essential for the morphinomimetic potency.

Since the biological activity involves not only the binding to the receptor but also the metabolic stability, it is important to dissociate the chemical modifications which are implicated in the 2 events.

The main degradation process by the aminopeptidases cleaves the amide bond between tyrosine and glycine [10]. The replacement of glycine by D-alanine decreases therefore the degradation of the pentapeptides without important change of the binding to the 2 kinds of receptors [12,13]. Thus, the correlative studies between the physico–chemical parameters and the biological activity of (D-Ala^2) analogues can be considered more useful to understand the essential parts for the binding to each kind of opiate receptors.

Two different conformations for the acid forms and the amide derivatives cannot be only deduced from the apparent differences in chemical shifts of some resonances, for example in the amide region (fig.1), since chemical shifts are known to be sensitive to charge effects as well as to conformational effects [14]. However, one of the most pervasive features of proton NMR spectra of enkephalin analogues is the important change in the $^3J_{NH-H\alpha}$ coupling constant values, particularly in the cases of the Met^5 analogues where we observe variations of 1.5 Hz for D-Ala^2 and Met^5 residues between acid and amide forms and, to a lesser extent, in the N-Val^5 analogues for the valine residue (0.7 Hz). A set of absolute conformations can be obtained from the values of the coupling constant $^3J_{NH-H\alpha}$ with the help of the Neel's graphs [15]; but we are interested, in a first step, in a more relative approach of the problem. If absolute $^3J_{NH-H\alpha}$ values alone are not sufficient to determine the backbone conformation of either the acid or amide forms of the 2 analogues, however, the observed variations in the coupling constants combined with the change in the chemical shifts clearly reflect differences in the conformational states of the acid and amide forms of the 2 analogues. Results on temperature-dependent chemical shift show that the Phe^4 amide proton exhibits a quite small coefficient (<2 ppb/degree) in the acid form indicating a solvent shielding environment and possible hydrogen bonding. This has also been observed in [16]. On the other hand this proton shows a normal exposed value (>3 ppb/degree) in the amide form.

These unambiguous data indicate obvious differences in the conformational states of the analogue

depending on the acidic or amidic forms of the C-terminal carboxylic function.

The question of the flexibility and preferential (folded or extended) conformation now arises. Self aggregation and equilibrium between folded and extended conformations must be considered at the concentration used on this study (14×10^{-3} M) [17,18]. Low concentrations ($\leq 10^{-3}$ M) greatly diminish self-aggregation but lead to ill-resolved spectra. Investigations on concentration-dependence showed that spectra run over 10^{-1}–10^{-3} M exhibited the same general features [8]; this allows us such a comparative study with minimal effects of self-association at $>10^{-3}$ M. 'Head-to-tail' interactions are well known for zwitterionic forms of the enkephalins and their carboxylic acid analogues. The mobility of the side chains of the tyrosyl and phenylalanyl residues can transfer to the peptidic backbone a relative flexibility, leading to possible conformational averaging. The lack of resonance for the phenolic proton of tyrosine, at low field of the spectrum, resulting from a rapid exchange with the protons of water present in the solution can be explained by a 'self-catalysis' mechanism, occurring through the carboxylic function, allowed by the flexibility of the molecule.

Substitution of the Gly^2 residue may lead to some conformational rigidity; (D-Ala^2, Met^5-OH) was found to have a 'relatively rigid backbone' [10]. This agrees well with the weak but significant presence of the Tyr-OH peak in the low field region of the spectra resulting from a less rapid exchange.

A predominant role may be played by the C-terminal carboxylic amide function in the rigidity of the peptidic backbone. The presence of the 2 C-terminal amide protons rules out the classical 'head-to-tail' interactions and can favour different and quite possible intramolecular interactions involving one of the 2 different amide protons. The resulting sharpness of the Tyr-OH signal in the N-Val^5 amide analogue spectrum (fig.1) occurs through a slow rate of exchange. This fact can be interpreted by considering a protected position of the phenolic proton buried inside a possibly folded structure of the overall molecule but we prefer, on the basis of molecular model-building and steric hindrance considerations to interpret this in terms of preferential orientation of the Tyr-OH proton induced by a possible folded and rigidified structure.

In [19] the amidation of the C-terminal part considerably modified the pharmacological activity on the 2 types of opiate receptors. The acidic character of the enkephalin analogues was necessary for strong interaction with δ receptors [19].

We report here that the effect of the amidation is more complex and that an increase of rigidity of the peptidic backbone can be considered. Such rigidity strongly increases the activity on μ receptors whereas it decreases the potency on δ receptors [20]. The increase of rigidity could explain the pharmacological variations of the biological activity which lead to the increase of the μ agonist activity and to the decrease of the δ agonist potency.

References

[1] Beddell, C. R., Clarck, R. B., Hardy, G. W., Lowe, L. A., Uba Tuba, F. B., Vane, J. R., Wilkinson, S., Chang, K. J., Cuatrecasas, P. and Miller, R. J. (1977) Proc. R. Soc. London B198, 229–265.

[2] Audigier, Y., Mazarguil, H. and Cros, J. (1980) FEBS Lett. 110, 88–90.

[3] Roques, B. P., Gacel, G., Fournier-Zaluski, M. C., Senault, B. and Lecomte, J. M. (1979) Eur. J. Pharmacol. 60, 109–110.

[4] Audigier, Y., Mazarguil, H., Gout, R. and Cros, J. (1980) Eur. J. Pharmacol. 63, 35–46.

[5] Audigier, Y., Mazarguil, H., Gout, R. and Cros, J. (1980) Eur. J. Med. Chem. 15 (2), 173–177.

[6] Kosterlitz, H. W. and Watt, A. J. (1968) Brit. J. Pharmacol. 33, 266–276.

[7] Hughes, J., Kosterlitz, H. W. and Leslie, F. M. (1975) Brit. J. Pharmacol. 53, 371–381.

[8] Mazarguil, H., Gairin, J. E. and Haran, R. (1981) unpublished.

[9] Shienblatt, M. I. (1966) J. Am. Chem. Soc. 88, 2123–2126.

[10] Niccolai, N., Garsky, V. and Gibbons, W. A. (1980) J. Am. Chem. Soc. 102, 1517–1520.

[11] Hambrook, J. M., Morgan, B. A., Rance, M. J. and Smith, C. F. C. (1976) Nature 262, 782–783.

[12] Pert, C. B., Pert, A., Chang, J. K. and Fong, B. T. W. (1976) Science 194, 330–332.

[13] Kosterlitz, H. W., Lord, J. A. H., Paterson, S. J. and Waterfield, A. A. (1980) Brit. J. Pharmacol. 68, 333–342.

[14] Jones, C. R., Garsky, V. and Gibbons, W. A. (1977) Biochem. Biophys. Res. Commun. 76, 619–625.

[15] Cung, M. T., Marraud, M. and Neel, J. (1974) Macromolecules 7, 606–613.

[16] Garbay-Jaureguiberry, C., Baudet, J., Florentin, D. and Roques, B. (1980) FEBS Lett. 115, 315–318.

[17] Khaled, M. A., Long, M. M., Thompson, W. D., Bradley, R. J., Brown, G. B. and Urry, D. W. (1977) Biochem. Biophys. Res. Commun. 76, 224–231.

[18] Higashijima, T., Kobayashi, J., Nagai, U. and Miyazawa, T. (1979) Eur. J. Biochem. 97, 43–57.

[19] Ronai, A. Z., Szekely, J. I., Berzetei, I., Miglecz, E. and Bajusz, S. (1979) Biochem. Biophys. Res. Commun. 91, 1239–1249.

[20] Lord, J. A. H., Waterfield, A. A., Hughes, J. and Kosterlitz, H. W. (1977) Nature 267, 495–499.